

AMENDMENTS

In the Specification:

Please replace the paragraph at page 5, line 16, through page 6, line 5, with the following:

-In yet another aspect of the present invention, a screening assay is provided for identification of small molecules that modulate frizzled receptor activity, which comprises:

- a) selecting a library of the small molecules comprising a plurality of different chemical structures;
- b) contacting the small molecules with an extracellular domain of a frizzled receptor which is capable of binding to its corresponding Wnt protein; and
- c) measuring binding of a ligand to the frizzled receptor in the presence of the small molecule, wherein the ligand is selected from the group consisting of the small molecule, the b) contacting the small molecules with an extracellular domain of a frizzled receptor which is capable of binding to its corresponding Wnt protein, and an antibody that is specific for the frizzled receptor epitope to which the Wnt protein normally binds from binding thereto. These types of screening methods are well known in the G-protein coupled receptor field, and in particular the field of odorant receptors. See, e.g., U.S. Patent No. 6,008,000, which discloses assays for screening taste modulating small molecules that modulate the activity of a G-protein coupled receptor known to be associated with taste.--

Please replace the paragraph at page 6, lines 10 to 13 with:

-Figure 2 depicts the alignment of various deduced amino acid sequences of frizzled receptors derived using the Clustal W program on DeCypher. "CRD" refers to the Cysteine Rich Domain. "TM" refers to the transmembrane domain. Accordingly, the regions in-between the CRD and TM domains represent the extracellular regions.--

Please replace the paragraph at page 6, line 23, through page 7, line 2, with :

C3
- Figure 8 depicts the sequence alignment of the deduced amino acid sequences of human (HFZ) and mouse (MFZ) frizzled receptors 1 to 10, assigned Seq. ID Nos. 44 to 60 in the order shown. Also depicted therein are the amino terminal domains (assigned Seq. ID Nos. 61 to 77 in the order shown), the extracellular domain loop 1 (assigned Seq. ID Nos. 78 to 94 in the order shown), the extracellular domain loop 2 (assigned Seq. ID Nos. 95 to 111 in the order shown), and the extracellular domain loop 3 (assigned Seq. ID Nos. 112 to 128 in the order shown)--

Please replace the paragraph at page 10, lines 1 to 9 with the following:

C4
- To evaluate frizzled receptors for their potential as tumor-associated antigens, various hematologic and epithelial tumors are screened by amplifying the mRNA in the tumor cells using a known amplification method, such as reverse-transcription-polymerase chain reaction (RT-PCR) using primers that are specific for known frizzled receptor-associated sequences. From the results of this initial screening, subregions of the nucleic acid sequence are identified that encode the extracellular regions of the frizzled receptor and are further amplified. The sequence alignment of a portion of the first extracellular region is shown in Figure 3. This extracellular amino terminal domain is generally regarded as antigenic, because of its size and tertiary structure--

Please replace the paragraph at page 15, lines 17 to 23, with the following:

C5
- The PCR buffer contains the deoxyribonucleotide triphosphates (i.e., polynucleotide synthesis substrates) dATP, dCTP, dGTP, and dTTP and a polymerase, typically thermostable, all in amounts sufficient for the primer extension (i.e., polynucleotide synthesis) reaction. An exemplary PCR buffer comprises the following: 50 mM KCl; 10 mM Tris-HCl at pH 8.3; 1.5 mM MgCl₂; 0.001% (wt/vol) gelatin, 200 microMolar (μ M) dATP, 200 μ M dTTP, 200 μ M dCTP, 200 μ M dGTP, and 2.5 units *Thermus aquaticus* (Taq) DNA polymerase I (U.S. Pat. No. 4,889,818) per 100 microliters (μ L) of buffer.--

Please replace the paragraph at page 22, lines 10 to 16, with the following:

The pharmaceutical compositions of the present invention include therapeutically effective amount of the appropriate anti-Fz Ab in a pharmaceutically acceptable carrier. Such carriers are well known in the art. Examples of appropriate carriers are those that are known for delivery of interferons, such as normal saline, dextrose, etc. The mode of administration of the pharmaceutical composition necessarily depends on the type and location of the target tumor cells. Accordingly, the compositions can be delivered, e.g., parenterally, or typically intravenously in a solution, suspension or emulsion.

Please replace the paragraph at page 22 line 24 to page 23 line 6 with the following:

For administration to an animal or human subject, the solutions are necessarily prepared to meet all FDA Office of Biologics standards. As such, they are normally dialyzed to remove undesired small molecular weight molecules or lyophilized with other active and excipient ingredients for reconstitution prior to administration. As would be appreciated by one of skill in the art, the administration parameters, such as dosage and timing, will necessarily depend on the type and location of the metastases to be treated and would easily be determined using routine optimization principles based on other like immunotherapeutics. Routes of suitable administration may include injection, intravenous, intramuscular, subcutaneous, intralesional, and the like. Alternatively, the immunotherapeutics of the present invention can be formulated for other local routes of administration as topicals, inhalants, orthotopic, ophthalmic, and the like.

Please replace the paragraph at page 23 lines 14 to 17 with the following:

The course of treatment may be monitored using appropriate immunoassays. For example, the level of circulating anti-Fz Abs following administration can easily be monitored using labeled anti-immunoglobulin antibodies in any of a number of commercially available assay formats.

Please replace the paragraph at page 23 line 25 to page 24 line 7 with the following:

To evaluate frizzled receptors for their potential as tumor associated antigens, the mRNA from various hematologic and epithelial tumors were screened, as well as the mRNA from normal cell lines. In this example total RNA was extracted from HNSCC lines (PCI13, Detroit 562, RPMI 2650, SNU1076, KB, AMC4), a CLL line (Lesch), a Burkitt lymphoma line (Ramos), glioma lines (U87MG, and U373MG), normal human bronchial epithelial cell lines (Clonetech, San Diego, CA) and normal oral squamous epithelial (OSE) cells using Trizol® Detroit 562, RPMI 2650, SNU1076, KB, AMC4), a CLL line (Lesch), a Burkitt lymphoma line (Ramos), glioma lines (U87MG, and U373MG), normal human bronchial epithelial cell lines (Clonetech, San Diego, CA) and normal oral squamous epithelial (OSE) cells using Trizol® (Gibco, BRL, Grand Island, New York). Reverse transcription was performed using 1 μ g of RNA from each sample and the Superscript™ Preamplification kit (Gibco BRL). Different pairs of gene-specific primers based on sequences of cloned human isoforms of the frizzled genes were used for reverse transcriptase-PCR (RT-PCR) analysis.

Please replace the paragraph at page 25 lines 9 to 20 with the following:

To determine the amount of protein expressed in the cells studied in Example 1, adherent cells in culture were harvested and lysed with a solution containing 25 mM Tris HCl, 150 mM KCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, 1mM NaVO₃, 1 mM NaF, 20 mM β -glycerophosphate and protease inhibitors. Twenty μ g of protein from each cell line was separated by SDS-PAGE and transferred to a PVDF membrane. The membrane was immersed in 2% I-block, 0.05% Tween X in PBS and then incubated with a 1:500 dilution of polyclonal goat anti-human frizzled 2 IgG (Santa Cruz Biotechnology, Santa Cruz, CA). These primary antibodies were then detected by horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz) and chemiluminescence (ECL detection reagents, Amersham Life Science, Aylesbury, UK). To verify relative amount of protein transferred in each lane, the presence of actin was measured with an actin monoclonal antibody (Chemi-Con International Inc, Temecula, CA).

Please replace the paragraph at page 26 lines 6 to 13 with the following:

--The ability to block the Wnt-frizzled signaling pathway can provide an effective way of limiting growth of tumor cells. In order to determine the efficacy of using such anti-Fz Abs as an adjunctive passive immunotherapy, such as that observed using humanized anti-HER2 antibodies (Herceptin, Genentech, Inc., South San Francisco, California), the effects of anti-frizzled 2 antibodies on the growth of HNSCC cells was studied. Soluble inhibitors of frizzled receptors have been described to induce apoptosis secondary to their inhibition of frizzled signaling. Accordingly, this experiment was designed to test the efficacy of anti-Fz Abs to perform the same function.

Please replace the paragraph at page 26 lines 14 to 24 with the following:

--Cell proliferation was determined by a colorimetric MTT-based assay. Briefly, either 7.5×10^3 or 10×10^3 SNU1076 cells per well were cultured in a 96 well plate. After 24 hours, graded amounts of polyclonal goat anti-human frizzled-2 antibody containing 300 ng, 30 ng, 3 ng, and 0.3 ng were added in the culture medium. The same concentrations of goat serum or Goat antihuman IgG (Fisher Scientific, Pittsburgh, PA) were used as controls. On 1, 2, 3, or 4 days after incubating antibody, 20 ul of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide)-based solution was added to the wells for four hours prior to lysis with 15% SDS, 0.015 M HCl. Absorbancies at 570 and 650 nm were measured. The results are depicted in Figure 4 and also given in Table IV below. Data represent the normalized growth fraction of the specific antibody treated cells to that of the control antibody treated cells (in triplicate).

Please replace the paragraph at page 27 lines 16 to 20 with the following:

--As shown in Figure 5, cells were detached from the flasks by trypsin treatment and incubated for 10 minutes in growing medium with 5 $\mu\text{g}/\text{ml}$ Propidium iodide and 40 nM DiOC₆ and analyzed by flow cytometry. Viable cells (Alive, right bars) had high DiOC₆ (FL-1) and low PI (FL-3) fluorescence, while apoptotic cells (left bars) had low DiOC₆ (FL-1) and low PI (FL-3) fluorescence.

Please replace the paragraph at page 28 lines 13 to 20 with the following:

14
C
For example, a panel of tumor cells that can be screened are derived from the panel of 60 lines which are being characterized in the NIH Developmental Therapeutics Program. The cell lines that are currently available in the lab include: (non-small cell lung cancer) A549/ATCC, NCI-H226, NCI-H460, HOP-62, HOP-92, (colon cancer) HT29, HCT-116, (breast cancer) MCF7, NCI/ADR-RES, MDA-MB-231/ATCC, T-47D, (ovarian cancer) OVCAR-3, OVCAR-4, SK-OV-3, (leukemia) CCRF-CEM, K-562, MOLT-4, HL-60(TB), RPMI-8226, (renal cell) 786-0, TK-10, (prostate cancer) PC-3, DU-145. Normal control cell lines will be purchased as previously from Clonetics.

Please replace the paragraph at page 29, lines 7-23 with the following:

15
C
Several plasmids have been constructed which are under the control of the cytomegalovirus (CMV) promoter which has been found to enable high levels of antigen expression in injected muscle. The pCMVint vector includes the cytomegalovirus (CMV) E1 promoter, the simian virus (SV40) t-intron, and the SV-40 polyadenylation site. The ACB vector has the same elements except the polyadenylation sequence is from the bovine growth hormone gene. For example, a preferred plasmid construct for frizzled-2 encodes the least homologous region of the frizzled gene between the ninth and tenth cysteine. These cysteines stabilize a configuration that enables antibody binding to the native protein. This polypeptide fragment is fused at the amino terminus or the carboxylterminus via a short linker to a tetanus toxin or measles MVF T helper epitope (see below). These minigenes are constructed with overlapping oligonucleotides. The oligonucleotides are 5' prime phosphorylated with T4 kinase (Boehringer Mannheim, Indianapolis, IN) at room temperature for 30 minutes, annealed by boiling an equimolar admixture of two complementary oligomers and slow cooling. The double stranded oligonucleotides are then ligated 3' to the tissue plasminogen leader (TPA) leader into the EcoR47III site in frame and into the BamHI site of the pBluescript SKII vector. The minigene is then subcloned into the pCMV and pACB vectors between the Pst1 and Xba1 sites as previously described.

Please replace the paragraph at page 32, lines 1-6 with the following:

C 1.6

- Plasmid DNA is prepared using Qiagen Maxiprep (Chatsworth, CA) kits with the modification of adding one tenth volume 10% Triton X-114 (Sigma, St. Louis, MO) to the clarified bacterial lysate prior to applying it to a column. Prior to injection the residual endotoxin level is quantified using a limulus extract clot assay (Associates of Cape Cod, Woods Hole, MA). A level of ≤ 5 ng endotoxin/ μ g DNA need be obtained prior to use in an animal. The DNA is resuspended in a sterile pyrogen free saline solution for injection.

Please replace the paragraph at page 32 lines 24 to 27 with the following:

C 1.7

- Another group of mice in similar groups is immunized using the pMMVF-FZD2 and pFZD2-MMVF set of linked epitope plasmids). The nCMVB7-1 and nCMVB7-2 constructs encode the cDNAs for murine CD80 and CD86 (provided by G. Freeman (Dana-Farber Cancer Institute, Boston, MA)).

Please replace the paragraph at page 33 line 19 to page 34 line 5 with the following:

C 1.8

- Once antibodies have been identified that delay cancer cell growth in cell culture, the ability of these antibodies can be tested for potential *in vivo* efficacy in mice. For example, the H-2^b thymoma line EL4 can be used as a syngeneic tumor in C57Bl/6 mice. This line is transfected with a human frizzled expression vector and selected in neomycin. The expression vector is made by excising the frizzled containing insert from a pET3a bacterial expression vector with NdeI and BamHI and ligating the insert into pcDNA3 which has a CMV promoter and a neomycin selection cassette. Thirty two female C57Bl/6 mice are divided into groups of 8 mice each. They are injected in the dermis of the tail with a combination of 50 μ g plasmid encoding a costimulator and 50 μ g linker plasmid diluted in normal saline at weeks zero, one and two. A group with empty vector is included as a negative control. On day 28 the mice are injected with 20×10^6 frizzled transfected EL4 cells or untransfected cells. The mice are monitored three times a week for weight, and tumor growth measured with a caliper. Tumor volume is calculated by length x width² x $\pi/6$. Mice are sacrificed four weeks post tumor challenge or if the tumor burden reaches approximately 2000mm³. Inhibition of tumor growth is determined by ANOVA.